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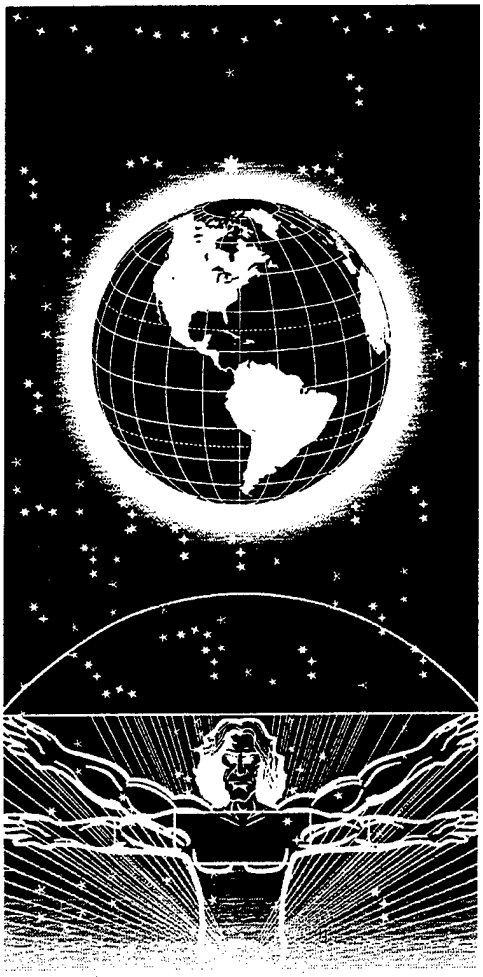

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

EFFECTS OF AMMONIUM DINITRAMIDE ON PREIMPLANTATION EMBRYOS IN SPRAGUE-DAWLEY RATS AND B6C3F1 MICE

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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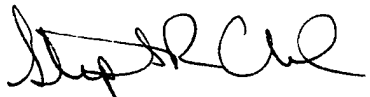

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The Department of Defense is considering replacing ammonium perchlorate (AP) with ammonium dinitramide (ADN) in rocket propellants and explosives. Previous studies performed at the Toxicology Division have shown that ADN is a female reproductive toxicant, causing implantation failure in Sprague-Dawley rats when administered during the preimplantation period of gestation. The purpose of this follow-up study was to identify the mechanism(s) associated with implantation failure following exposure to ADN. In Phase I, mated female rats were treated with 2.0 grams/liter (g/L) ADN in their drinking water for 24, 48, 72, or 96 hours before preimplantation embryos were harvested from the oviducts or uterine horns. On gestation day (GD) 1, comparable numbers of morphologically normal 2-cell embryos were harvested from the oviducts of the treatment and control groups. On GD-2, the development of the embryos harvested from the treated animals was either slowed or halted when compared to the control embryos. By GD-4, 98% of the embryos harvested from the control group had developed to the morula or blastocyst stage; these were collected from the uterine horns. On GD-4 in the treated group, 41% of the harvested embryos remained at the 2- to 6-cell stage and 59% were degenerate; 82% of these embryos were collected from the oviducts. In Phase II, 2-cell embryos were harvested from the oviducts of superovulated mated B6C3F1 mice. The embryos were cultured for 72 hours in medium supplemented with 0 mM, 1 mM, 4 mM, 6 mM, 10 mM, or 20 mM ADN. The development of embryos *in vitro* was affected by ADN in all dose groups when compared to the control group. Culture in medium containing > 1 mM ADN either slowed or arrested development of the embryos.

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PREFACE

This is one of a series of technical reports describing results of experimental laboratory programs conducted at the Toxicology Division under the ManTech Geo-Centers Joint Venture Toxic Hazards Research Contract. This document serves as a final report on the preimplantation effects of ammonium dinitramide administered in the drinking water of Sprague-Dawley rats. The research described in this report began in January 1995 and was completed in March 1996 under Department of the Air Force Contract Nos. F33615-90-C-0532 and F4164-96-C-9010. Lt Col Terry A. Childress served as the Contracting Officer's Representative for the U.S. Air Force, Armstrong Laboratory. Darol E. Dodd, Ph.D., served as Program Manager for the ManTech Geo-Centers Joint Venture.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication No. 86-23, 1985, and the Animal Welfare Act of 1966, as amended.

The authors gratefully acknowledge Marcia L. Feldmann, Jerry W. Nicholson, and Margaret A. Parish for their excellent technical assistance.

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ABBREVIATIONS

ADN	Ammonium Dinitramide
AP	Ammonium Perchlorate
Blast	Blastocyst
C	Celsius
DOD	Department of Defense
GD	Gestation Day
g/L	Gram/liter
HB	Hatching Blastocyst
HCG	Human Chorionic Gonadotropin
IU	International Units
IP	Intraperitoneally
kg	Kilogram
mL	Milliliter
mM	millimolar
N	Number
P	Probability
SEM	Standard error of the mean
SD	Standard deviation
wt/vol	Weight per volume

SECTION I

INTRODUCTION

The Department of Defense (DOD) is working towards compliance with international standards in minimizing the use of ozone depleting substances. The DOD is considering replacing ammonium perchlorate (AP) with ammonium dinitramide (ADN) in rocket propellants and explosives. AP leaves a heavy hydrochloric acid smoke trail upon burning. The chlorine has been identified as an ozone depleting substance, and the smoke trail makes rockets more vulnerable to detection. ADN is a clean burning more efficient compound. Preliminary data has shown that ADN is a female reproductive toxicant in rats. Additional *in vivo* and *in vitro* studies are required to determine the mechanism(s) associated with the compound's reproductive toxicity. The data obtained from these studies will be valuable to military industrial hygiene officers, as well as to hygienists responsible for personnel at production facilities.

Background Reproductive Toxicity Data Of ADN

Previous studies have shown that ADN is a female reproductive toxicant in Sprague-Dawley rats. A 90-day reproductive toxicity screen of ADN administered in the drinking water of Sprague-Dawley rats showed that only 3 of 12 mid-dose (103 mg ADN/kg/day) dams and 1 of 12 high-dose (162 mg ADN/kg/day) dams produced live litters, compared to 9 of 12 in the control group and 11 of 12 in the low-dose (29 mg ADN/kg/day) group (Kinkead et al.,1994). A follow-up study, a pre-vs. post-implantation protocol, indicated that ADN exerts its effects during the preimplantation period in rats,

or gestation days (GD) 1-3 (Kinkead et al., 1995b). If ADN is administered in drinking water during this period at a concentration of 2.0 g/L, implantation is totally blocked. If the same concentration of the compound is administered only on GD 4-8, the number of implantation sites is comparable to the control group. Mean doses for the two groups were 211 and 199 mg/kg/day, respectively. The number of corpora lutea was consistent in each group, implying that ovarian function was not affected by ADN treatment. Serum progesterone, prolactin, and estradiol were affected by ADN treatment ($p < 0.01$) when compared to the control group. These hormone levels are also driven by maternal signals and normal implantation/embryonic development, so these data are somewhat ambiguous. The cause of implant failure could occur at a number of other points during the reproductive process: 1) oviduct motility which affects sperm, ova, and embryo transport, 2) uterine receptivity, and 3) ova/embryolethality. This study was designed to investigate the cause of the implantation failure from a mechanistic point of view. In Phase I, female rats received ADN-treated drinking water beginning on GD-0. A control and treatment group were necropsied on GD 1, 2, 3, and 4; the location and stage of development of the embryos were evaluated. In Phase II, 2-cell murine embryos were cultured in ADN supplemented medium. Embryonic development was evaluated over a 72-hour period.

SECTION II

MATERIALS AND METHODS

Test Compound

The ADN [$NH_4N(NO_2)_2$] was supplied by SRI International, Menlo Park, CA. ADN is a white soluble powder. Because the test compound is light sensitive, it was stored in protective vials in an enclosed cabinet. The test compound was known to be contaminated with 1-2% ammonium nitrate (Koppes, 1993).

The test compound drinking water solution was prepared by adding a specific amount of ADN to a known amount of reverse osmosis-treated water (wt/vol), as described by Kinkead et al., 1995a. The test solution was light-protected during use.

Animal Husbandry

Phase I

Male and female Sprague-Dawley rats, 11 weeks of age, were purchased from Charles River Breeding Laboratories, Raleigh, NC. All animals were single housed, identified by tattoo, and allowed a 14-day acclimation period. Water and feed (Purina Formulab #5002, St. Louis, MO) were available *ad libitum* prior to treatment. The females' drinking water was supplied via glass water bottles equipped with stainless steel sipper tubes and neoprene stoppers. The animal room temperature was maintained at 21-25 °C and the light/dark cycle was set at 12-hour intervals. During the mating period, the animals were housed in clear plastic cages with stainless steel wire bottoms.

Phase II

Female B6C3F1 (23 days of age) and male CD-1 (42 days of age) mice were purchased from Charles River Breeding Laboratories, Raleigh, NC. All animals were single housed, identified by tattoo, and allowed a 14-day acclimation period. Water and feed (Purina Formulab #5002, St. Louis, MO) were available *ad libitum*. The animal room was maintained at 21-25 °C and the light/dark cycle was set at 12-hour intervals.

Study Design

Phase I

The embryo transport technique developed by Cummings (1990) was employed in Phase I. The animals were mated one pair/cage; the observance of a copulatory plug was designated as GD-0. Treatment of the females was begun immediately following observation of a copulatory plug. The drinking water for the treated groups was supplemented with 2.0 grams ADN per liter drinking water. The mated females were randomly assigned to one of 8 groups (n= ≥ 5/group): control or treatment GD-1; control or treatment GD-2; control or treatment GD-3; or control or treatment GD-4. The animals were sacrificed via CO_2 inhalation on the gestation day corresponding to their group assignment. The oviducts and uterine horns were removed to separate culture dishes and their lumens flushed with phosphate-buffered saline to expel the embryos. The embryos were collected, counted, their location (oviduct or uterus) recorded, and the stage of development evaluated via phase contrast microscopy.

Phase II

The females were injected intraperitoneally (IP) with 10 International Units (IU) pregnant mare's serum gonadotropin (Sigma, St. Louis, MO) followed 48 hours later by an IP injection of 10 IU human chorionic gonadotropin (HCG, Sigma, St. Louis, MO). The animals were mated one pair/cage at the time of HCG injection. The females were sacrificed 38 hours later by cervical dislocation. Under aseptic conditions, the oviducts were removed to culture dishes and flushed with medium. The harvested 2-cell embryos were placed into culture in control medium or medium supplemented with 0, 1mM, 4mM, 6mM, 10mM, or 20mM ADN. The development of the embryos was monitored via phase contrast microscopy daily for 72 hours.

Statistical Analysis

Phase I

The statistical method used was Multiway Frequency Tables (Dixon, 1993). The factors were treatment (treated or untreated), stage of embryonic development, and gestation day. The statistical distribution was analyzed by the Chi-squared test.

Phase II

The statistical method used was Multiway Frequency Tables (Dixon, 1993). The dependent variable (stage of embryonic development) was a multiway nomial. The independent variables were number of hours in culture and concentration of the test compound. The statistical distribution was analyzed by the Chi-squared test. Individual stages were

combined based on the zero (0) treatment and day. Bonferroni multiple comparisons were performed (Fleiss, 1981).

RESULTS

Phase I

The animals were necropsied 24, 48, 72, or 96 hours after initiation of treatment; there were no significant differences in the body weights of each treatment group when compared to the respective control group. Only treated animal water consumption was measured in this study (Table 1), but control female rat water consumption historically averages 35 mL/day (Kinkead et al., 1994). ADN consumption is shown in Table 2. Water consumption and ADN dosages did not differ significantly from those previously reported for studies performed in this laboratory (Kinkead et al., 1994, 1995a, 1995b). There was a significant difference in embryonic development for each treatment group compared to its control group (Table 3). On GD-1, 7% of the embryos harvested from treated animals were degenerate compared to 0% in the control group. On GD-2, 79% of the control embryos had reached the 3-to 6-cell stage; 16% remained at the 2-cell stage. In the GD-2 treated group, 20% of the embryos had reached the 3-to 6-cell stage, while 49% remained at the 2-cell stage. This trend continued on GD-3. By GD-4, 98% of the control embryos had developed to the morula or blastocyst stage; these were harvested from the uterine horns. In the treatment group, 59% of the embryos were degenerate and 41% remained at the 2-to 6-cell stage; 82% of these embryos were harvested from the oviducts (Table 4).

Statistical Analysis

The Chi-squared for days 1-4 ($p = 0.0298$, 0.0000, 0.0000, and 0.0000, respectively) indicated a significant difference in embryonic development between the treated and control groups for each gestational day.

Phase II

The development of murine embryos cultured *in vitro* was affected by all concentrations of ADN (Table 5). Overall, 10.6 ± 0.70 (SEM) 2-cell embryos were harvested per dam. These were randomly distributed in culture among the doses. In the control cultures, 77% of the embryos had reached the morula stage after 24 hours; 100% of the control embryos developed to the hatching blastocyst stage after 72 hours in culture. There were no degenerate embryos found in the control cultures. After 72 hours in culture, the development of the embryos cultured in 1 mM ADN lagged behind the control group by 24 hours. After 72 hours in culture, 67% of the embryos in the 4 mM group were blastocysts, 28% were morulas, and 3% were degenerate. After 72 hours in 6 mM ADN, 50% of the embryos remained at the 3-to 16-cell stage; 36% were morulas or blastocysts and 14% were degenerate. After 72 hours in 10 mM ADN, 44% of the embryos remained at the 4-to 16-cell stage, 32% were morulas, and 24% were degenerate. After 72 hours in the high-dose group, 20 mM ADN, 83% remained at the 2- to 16-cell stage and 17% were degenerate.

Statistical Analysis

The interaction among day, stage of embryonic development, and concentration of the test compound was significant ($p=0.0720$) indicating that both day and concentration affected the dependent variable (stage of embryonic development). After 24 hours in culture, there were significant differences between 0 and 20 mM ($p=0.0012$) and 1 and 20 mM ($p=0.0050$). After 48 hours in culture, there were significant differences between 0 and 6 mM ($p=0.0081$), 0 and 10 mM ($p=0.0035$), 0 and 20 ($p= 0.0019$), 1 and 6 mM ($p=0.0123$), 1 and 10 mM ($p=0.0054$), and 1 and 20 mM ($p=0.0031$). After 72 hours in culture, 0 mM differed from 1, 4, 6, 10, and 20 mM at $p < 0.0001$, and 1 mM differed from 6, 10, and 20 mM at $p < 0.0010$. Thus, embryonic development was both dose- and time- dependent.

DISCUSSION

The implantation process is a complex blend of events leading to the successful implantation of a blastocyst in the uterine lining. A normal estrus cycle must be followed by timely fertilization, normal development and transport of the zygotes through the oviducts, and the mounting of a decidual response by the uterus. Disruption of these events at any point may lead to implantation failure. In the rat, ova are fertilized within hours of mating; the embryos develop to the blastocyst stage in the oviducts during GD 0-4. The hatching blastocysts enter the uterine horns on GD-4 and implant between GD 4 and 5 (DeFeo, 1967).

In the pre- vs. post-implantation study, none of the dams treated during the preimplantation period had implantation sites, but the numbers of corpora lutea and the weight gain accompanying decidualization were comparable to the control group. These data imply that ovarian and uterine function were not affected by ADN treatment (Kinkead et al., 1995b). In the same study, serum progesterone, prolactin, and estradiol were significantly reduced ($p < 0.01$) at necropsy on GD-9 in the group that was treated with ADN during the preimplantation period (GD-0 to GD-3) implying that ADN may affect pituitary function, but normal levels of these hormones are also driven by maternal-embryonic signals and a normal implantation process (Weitlauf, 1988). Further investigation would be required to determine if ADN targets the endocrine system.

The data presented here indicate that embryolethality may be, at least in part, responsible for the implantation failure seen following treatment with ADN. In Phase I,

morphologically normal blastocysts were recovered from the uterine horns on GD-4 in the control group. In the treated groups, embryonic development was slowed or halted as early as GD-2. No embryos recovered from the treated groups had developed past the 4-cell stage. The mechanism by which ADN exerts its embryolethal effect is unknown. ADN is a known genotoxin (Zhu et al., 1994) and may be genotoxic to the developing embryo in the oviduct. It is also possible that the hormonal/biochemical milieu in the oviductal fluid may in some way be affected by ADN.

In Phase II, the development of embryos *in vitro* was affected by concentrations of ADN as low as 1 mM. There were significant differences in embryonic development after 24, 48, and 72 hours in culture. These differences increased over culture time, and were dose-dependent. These results correlate with viability studies done with hepatocytes cultured in similar concentrations of ADN (Dean and Channel, 1995). The results of that study suggested that ADN is a cytotoxic genotoxin, possibly indirectly damaging DNA through an oxidative challenge mechanism. Additional studies would be necessary to determine if the embryolethality reported in this study was the result of genotoxicity/oxidative stress.

TABLE 1. PHASE I DATA: WATER CONSUMPTION OF TREATED FEMALE RATS

Water Consumption (mL/day)

	Group:	GD-1	GD-2	GD-3	GD-4
Day 1	N	6	7	8	7
	Mean	27.7	25.7	26.6	28.0
	SD	5.4	5.1	4.0	3.4
	SEM	2.2	1.9	1.4	1.3
Day 2	N	N/A	5	8	7
	Mean	N/A	31.8	28.4	33.7
	SD	N/A	3.7	4.3	4.3
	SEM	N/A	1.6	1.5	1.6
Day 3	N	N/A	N/A	8	7
	Mean	N/A	N/A	32.9	31.1
	SD	N/A	N/A	5.2	4.2
	SEM	N/A	N/A	1.9	1.6
Day 4	N	N/A	N/A	N/A	6
	Mean	N/A	N/A	N/A	33.3
	SD	N/A	N/A	N/A	6.6
	SEM	N/A	N/A	N/A	2.7

TABLE 2. PHASE I DATA: ADN CONSUMPTION OF TREATED FEMALE RATS

ADN Consumption (mg/kg/day)

	Group:	GD-1	GD-2	GD-3	GD-4
Day 1	N	6	7	8	7
	Mean	216.4	190.7	204.6	209.3
	SD	52.2	37.3	32.6	21.4
	SEM	21.3	14.0	11.5	8.1
Day 2	N	N/A	5	8	7
	Mean	N/A	227.5	213.9	250.7
	SD	N/A	21.1	35.4	35.4
	SEM	N/A	9.4	12.5	12.5
Day 3	N	N/A	N/A	8	7
	Mean	N/A	N/A	242.2	227.3
	SD	N/A	N/A	42.3	26.2
	SEM	N/A	N/A	14.9	9.9
Day 4	N	N/A	N/A	N/A	6
	Mean	N/A	N/A	N/A	241.5
	SD	N/A	N/A	N/A	44.0
	SEM	N/A	N/A	N/A	18.0

TABLE 3. PHASE I DATA: NUMBER AND STAGE OF DEVELOPMENT OF HARVESTED EMBRYOS

Stage of Embryonic Development

	Groups	2 Cell	3-4 Cell	5-6 Cell	8-12 Cell	Morula	Blastocyst	Degenerate	Tot
GD-1	Treated	57	0	0	0	0	0	4	61
	Control	59	3	0	0	0	0	0	62
GD-2	Treated	32	13	0	0	0	0	20	65
	Control	10	41	7	2	0	0	1	61
GD-3	Treated	10	2	0	0	0	0	46	58
	Control	1	4	11	36	9	2	3	66
GD-4	Treated	10	11	0	0	0	0	30	51
	Control	0	0	0	0	12	49	1	62

TABLE 4. PHASE I DATA: LOCATION OF HARVESTED EMBRYOS

		Location of Harvested Embryos (Percentage of Total)	
	Groups	Oviducts	Uterine Horns
GD-1	Treated	100	0
	Control	100	0
GD-2	Treated	100	0
	Control		
GD-3	Treated	100	0
	Control	100	0
GD-4	Treated	82	18
	Control	0	100

TABLE 5. PHASE II DATA: NUMBER AND STAGE OF DEVELOPMENT OF *IN VITRO* CULTURED EMBRYOS

Hours in Culture	Dose (mM)	Stage of Embryonic Development						
		2 Cell	4-8 Cell	10-16 Cell	Morula	Blast	HB	Degenerate
24	0	0	0	7	24	0	0	0
	1	0	4	7	22	0	0	0
	4	0	11	7	15	2	0	0
	6	0	14	8	2	0	0	0
	10	0	17	6	1	0	0	0
	20	5	23	0	0	0	0	0
		2 Cell	4-8 Cell	10-16 Cell	Morula	Blast	HB	Degenerate
48	0	0	0	0	0	31	0	0
	1	0	1	0	1	31	0	0
	4	0	0	0	11	24	0	1
	6	0	3	14	5	1	0	1
	10	0	9	10	2	0	0	4
	20	4	23	0	0	0	0	0
		2 Cell	4-8 Cell	10-16 Cell	Morula	Blast	HB	Degenerate
72	0	0	0	0	0	0	31	0
	1	0	0	0	1	31	0	0
	4	0	0	0	10	24	1	1
	6	0	2	9	8	0	0	3
	10	0	4	7	8	0	0	6
	20	2	17	0	0	0	0	4

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